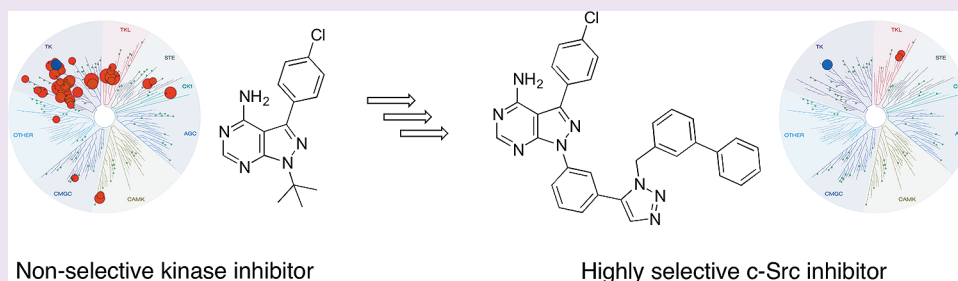# Development of a Highly Selective c-Src Kinase Inhibitor

Kristoffer R. Brandvold,[†] Michael E. Steffey,[†] Christel C. Fox,[†] and Matthew B. Soellner*[,†,‡]

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S *Supporting Information*

Non-selective kinase inhibitor ⟹ Highly selective c-Src inhibitor

ABSTRACT: Generating highly selective probes to interrogate protein kinase function in biological studies remains a challenge, and new strategies are required. Herein, we describe the development of the first highly selective and cell-permeable inhibitor of c-Src, a key signaling kinase in cancer. Our strategy involves extension of traditional inhibitor design by appending functionality proposed to interact with the phosphate-binding loop of c-Src. Using our selective inhibitor, we demonstrate that selective inhibition is significantly more efficacious than pan-kinase inhibition in slowing the growth of cancer cells. We also show that inhibition of c-Abl kinase, an off-target of most c-Src inhibitors, promotes oncogenic cell growth.

Post-translational phosphorylation is a key event within signaling pathways in eukaryotes.[1,2] Understanding the role of individual protein kinases within this process has been complicated by a lack of truly selective kinase inhibitors.[3,4] Generating selective probes for kinases remains a significant challenge because essentially all known kinase inhibitors function through competitive binding in a highly conserved ATP pocket.[3,5] While genetic techniques, including RNA interference, can inactivate specific genes, most kinases are multidomain proteins where each domain has an independent function.[1] Small molecules, however, can inhibit kinase catalytic activity without perturbing the other domains.

The non-receptor tyrosine kinase c-Src plays a vital role in many facets of cell physiology, regulating diverse processes including cell division, motility, adhesion, angiogenesis, and survival.[6,7] c-Src was the first proto-oncogene to be identified and is frequently overexpressed in cancers.[7] Furthermore, the extent of this overexpression typically correlates with malignant potential and patient survival.[6,7] Recently, c-Src was identified as the major resistance factor to Herceptin, a first line therapy for Her2+ breast cancer.[8] Despite the significant attention devoted to c-Src inhibitor discovery, there are no highly selective probes for c-Src suitable for chemical genetic experiments in native systems.[9−11]

To fully understand the role of c-Src in oncogenesis, specific probes of c-Src function are required. Herein, we describe the development of the first highly selective and cell-permeable inhibitor of c-Src. Our approach involves modifying an existing non-selective inhibitor to interact with an adjacent pocket formed by the phosphate-binding loop of c-Src. This approach represents an underutilized method for improving kinase inhibitor selectivity that is likely generalizable across many kinase families.[12] We have developed the most selective c-Src inhibitor to date, and using this inhibitor, we demonstrate that selective inhibition of c-Src is significantly more efficacious than multikinase inhibition in cell culture. Finally, using our probe we show that inhibition of a common off-target kinase of c-Src inhibitors, c-Abl, is pro-oncogenic in a breast cancer cell model.
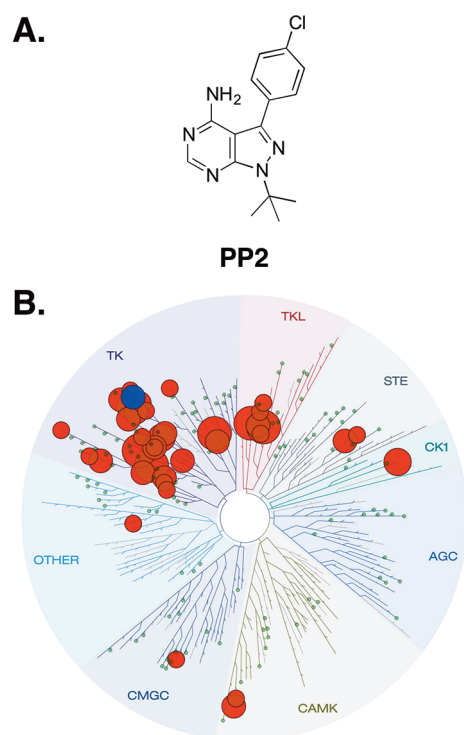
■ RESULTS AND DISCUSSION

Kinome Profiling of PP2. We started our work by examining PP2, a well-known inhibitor reported to be highly selective for c-Src.[13] The description of PP2 as selective arises from a 2007 report in which several kinase inhibitors were profiled against a panel of 73 kinases, most of which were Ser/Thr kinases.[14] Despite over 1,000 subsequent biological studies using PP2 as a tool, no broader kinome profiling of PP2 has been reported. To test PP2's selectivity more definitively, the inhibitor was screened against a diverse panel of 200 kinases using an *in vitro* ATP-site competition binding assay (KINOMEscan[15]) at a concentration of 10 μM. In contrast to previous reports,[14] PP2 is classed as non-selective from these data ($S_{35} = 0.41$, Figure 1). S_{35} is calculated by dividing the number of kinases with less than 35% of control by the total number of kinases tested. In the KINOMEscan panel, 56 kinases showed greater than 95% displacement from an

A.

PP2

B.
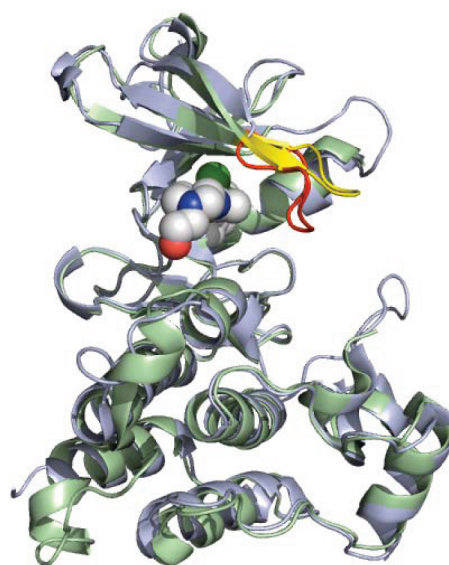

Figure 1. (A) Structure of PP2. (B) Kinome dendrogram of PP2 selectivity profiling at 10 μM. c-Src is colored blue, and off-target kinases of PP2 are colored red. Dendrogram was generated using TREEspot software tool with 10% cutoff. Green circles denote kinases kinases included in panel that show no binding below cutoff. Dendrogram reprinted with permission from KINOMEscan.

immobilized ligand by PP2 (24 kinases had >99% displacement). On the basis of our results, PP2 is less selective than dasatinib, a well-studied and promiscuous tyrosine kinase inhibitor (in this panel S_{35} = 0.27).[4] Given this lack of selectivity, interpretation of the results from the numerous reports using PP2 in biological studies are complicated by inhibition of many other kinases. For example, PP2 has been used to demonstrate that c-Src activity modulates ErbB2 and ErbB3 phosphorylation state in breast cancer.[16] However, our profiling shows that PP2 significantly inhibits both ErbB2 and ErbB3. For the complete profiling data for PP2, see the Supporting Information.
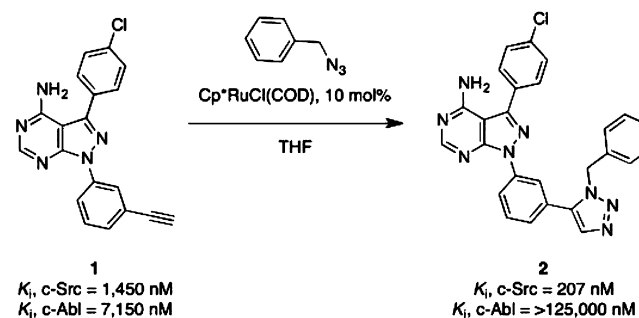
Design of Selective c-Src Inhibitor. Based on co-crystal structures of PP2 bound to c-Src and Src-family kinases,[17,18] we hypothesized that PP2 could be modified to obtain a selective inhibitor. Specifically, a pocket formed by the phosphate-binding loop (P-loop, also known as the glycine-rich loop) was identified in c-Src that did not appear in homologous kinases, including c-Abl (for molecular models of this pocket, see the Supporting Information). c-Abl is a tyrosine kinase with high sequence similarity to that of c-Src (69% across the entire kinase domain) and nearly identical ATP-binding pockets. No clinical or preclinical inhibitors of c-Src have been reported that do not also inhibit c-Abl.[4] Therefore, c-Abl was chosen as our initial test for inhibitor selectivity. From our analyses of co-crystal structures of dasatinib bound to both c-Src and c-Abl, c-Src has a more "open" P-loop compared to c-Abl (Figure 2). Likewise, in co-crystal structures of imatinib with c-Src and c-Abl the P-loop of c-Src is "open", whereas the P-loop of c-Abl is "closed" (Supporting Information Figure S1).
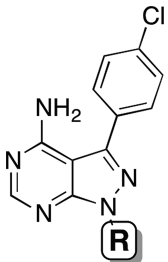

Figure 2. Alignment of structures with dasatinib bound to c-Src (PDB code: 3QLG) and c-Abl (PDB code: 2GQG). c-Src is colored light green with the P-loop of c-Src highlighted yellow. c-Abl is colored light blue with the P-loop of c-Abl highlighted red. Dasatinib is shown in space fill model.
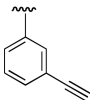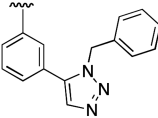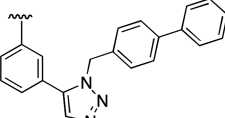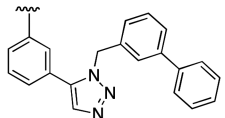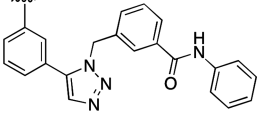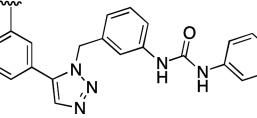
To obtain molecules that could interact with the P-loop pocket of c-Src, a PP2 analogue containing an aryl alkyne handle was synthesized. PP2~alkyne (**1**) is a modest inhibitor of c-Src (K_i = 1.4 μM). On the basis of our proposed molecular docking model (see Supporting Information Figure S2), PP2~alkyne scaffold was elaborated using benzyl azide and either Cu- or Ru-based click chemistry[19] to form 1,4- or 1,5-disubstituted benzyl triazoles, respectively. Consistent with our model, the 1,4-disubstituted benzyl triazole is not an effective inhibitor of c-Src (see Supporting Information), whereas the 1,5-disubstituted benzyl triazole (**2**) has increased binding affinity for c-Src (K_i = 207 nM). Significantly, compound **2** did not inhibit c-Abl activity up to 125 μM (Scheme 1).

Scheme 1. Synthesis and Biochemical Characterization of Benzyl Triazole Compound 2

1
K_i, c-Src = 1,450 nM
K_i, c-Abl = 7,150 nM

2
K_i, c-Src = 207 nM
K_i, c-Abl = >125,000 nM

In an effort to further improve potency, a limited number of analogues (compounds **3**−**6**) were synthesized (Table 1). From this series of compounds, *meta*-substituted biphenyl **4** was the only compound that had improved binding (lower K_i) compared to c-Src. Compound **4** is a potent inhibitor (K_i = 44 nM) of c-Src and does not inhibit c-Abl up to 125 μM. The binding affinity of compound **4** for c-Src is comparable to that

Table 1. Summary of K_i Values Obtained for Compounds 1–6

Compound	R =	K_i c-Src (nM)	K_i c-Abl (nM)
1		1,450	7,150
2		207	>125,000
3		>60,000	N.D.
4		44	>125,000
5		37,000	N.D.
6		>60,000	N.D.

A.

4

B.

Figure 3. (A) Structure of compound **4**. (B) Kinome dendrogram of compound **4** selectivity profiling at 10 μM. c-Src is colored blue, and off-target kinases of compound **4** are colored red. Dendrogram was generated using TREEspot software tool with 10% cutoff. Green circles denote kinases kinases included in panel that show no binding below cutoff. Dendrogram reprinted with permission from KINOMEscan.

Table 2. K_d Values Obtained by KINOMEscan for Src Family Kinases with Compound 4

kinase	K_d (nM)
c-Src	86
Lck	160
Fgr	240
Yes	720
Lyn	3200
Hck	4400
Fyn	>40,000

of PP2 (K_i = 33 nM). However, PP2 is also an effective c-Abl inhibitor (K_i = 325 nM).

Compound **4** was screened against a diverse kinase panel (KINOMEscan[15]) using an *in vitro* ATP-site competition binding assay at a concentration of 10 μM (Figure 3). From this panel, we found that compound **4** was remarkably selective. Only three kinases (c-Src, c-Raf, and B-Raf) exhibited >95% displacement from an immobilized ligand by compound **4** (only c-Src had >99% displacement). Detailed selectivity profiling results can be found in the Supporting Information.

Using this same method,[15] K_d measurements were obtained for c-Src and six homologous kinases that are members of the Src kinase family and not found in the panel (Table 2). Obtaining selectivity across this conserved kinase family has been a challenging task, with only a handful of compounds identified that can discriminate between them.[20,21] The K_d for c-Src (86 nM) was in good agreement with the K_i we obtained in our biochemical activity assay. Compound **4** is selective between Src family members, with >2-fold selectivity over both Lck and Fgr, 8-fold selectivity over c-Yes, and >40-fold selectivity over Lyn, Hck, and Fyn (Table 2).
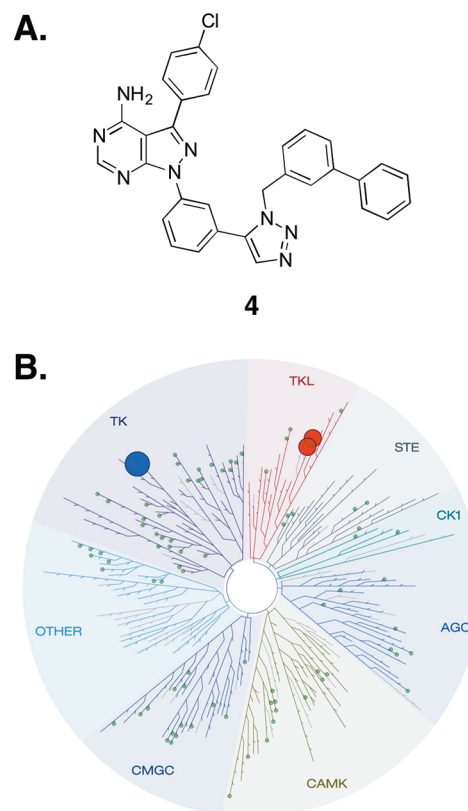
Interactions with the P-loop of kinases have previously been reported to modulate selectivity in kinase–ligand interactions.[22–24] Typically, these interactions are not due to the primary sequence of the P-loop, but rather due to distributed contributions throughout the kinase catalytic domain.[23] To probe the selectivity of compound **4**, the P-loop of c-Src (residues 273–281, chicken c-Src numbering) was replaced with the P-loop residues of c-Abl. Compound **4** bound c-Src-TM (Q275G, C277Q, F278Y) with potency similar to that of wild-type c-Src (K_i = 175 nM). This suggests that the primary sequence of the P-loop is not solely responsible for the observed selectivity. Studies aimed at better understanding compound **4**'s impressive selectivity are in progress.

Cellular Phosphorylation Assays. To serve as an effective probe in biological studies, the probe must function in cells.

Compound **4** was incubated with murine embryonal fibroblast (MEF) cells exogenously expressing full-length c-Src. The change in phosphorylation of c-Src Tyr-416 was measured using specific antibodies in a sandwich ELISA.[25] In this assay, compound **4** has an $IC_{50} = 1.9$ μM indicating that our probe is cell-permeable and inhibits c-Src activity in cells (Supporting Information Figure S2).

In the kinome profiling of compound **4**, B-Raf and c-Raf were inhibited along with c-Src. To assess whether compound **4** has Raf inhibitory activity *in cellulo*, phosphorylation changes in Erk, a downstream substrate of B-Raf and c-Raf, were measured. In SK-BR-3 cells stimulated with EGF to activate the Raf pathway, treatment of 100 μM compound **4** had no effect on p-Erk levels (see Supporting Information for details). From these results, we assume that cellular inhibition of B-Raf and/or c-Raf is not significant.[26]
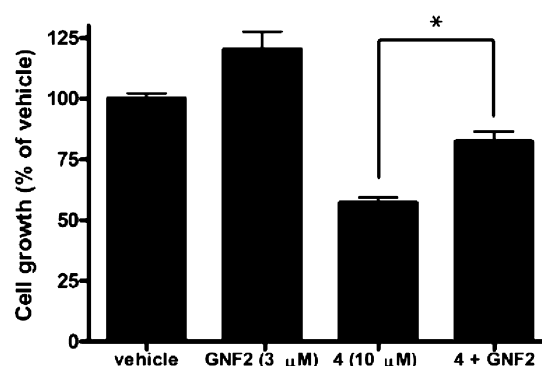
Inhibition of Cancer Cell Growth. We were interested in whether selective c-Src inhibition would be as efficacious as multikinase inhibition.[27] To test this, our selective probe **4** was compared to the non-selective inhibitor PP2. PP2 has a nearly identical biochemical K_i for c-Src and has been extensively used in cell culture experiments. Four different cancer cell lines were examined,[28] each of which has been shown to be growth-dependent upon c-Src activity.[29] In all cancer cell lines tested, compound **4** is more efficacious than PP2 (Table 3).

Table 3. Biochemical and Cellular Characterization of PP2 and compound 4

	PP2	compound 4
K_i, c-Src	0.033 μM	0.044 μM
GI_{50}, HT-29	48 μM	11 μM
GI_{50}, SK-BR-3	>100 μM	12 μM
GI_{50}, MCF7	>100 μM	11 μM
GI_{50}, MDA-MB-453	14 μM	6.0 μM
GI_{50}, NIH-3T3	17 μM	>100 μM

Addressing the debate about whether selective inhibition offers any advantage for kinase inhibitor therapeutics has been complicated by a lack of truly selective kinase inhibitors.[27] In our studies, selective inhibition leads to improved cellular efficacy.

The most profound differences between compound **4** and PP2 were found in cell lines derived from breast cancer tumors. Recent work has shown that, in contrast to its role in hematopoietic cancers, c-Abl activity in breast cancer is anti-oncogenic.[30] That is, inhibition of c-Abl in breast cancer increases disease progression. We hypothesized that the increased efficacy of compound **4** could be due, in part, to removal of c-Abl inhibition. To test this hypothesis, the efficacy of compound **4** was examined in 4T1 cells, which are frequently used as a late-stage model of metastatic breast cancer. Schiemann and co-workers have previously demonstrated that expression of a constitutively active c-Abl gene is sufficient to prevent growth of 4T1 cells in 3D culture.[31] The efficacy of c-Src inhibitors has not previously been reported in 4T1 cell culture; however, we found that 4T1 cell growth in 3D culture is dependent upon c-Src activity using compound **4** (Figure 4). To determine whether inhibition of c-Abl can mitigate the efficacy of compound **4**, a highly specific inhibitor of c-Abl, GNF-2,[32] was used. When GNF-2 and compound **4** are dosed together, the decrease in 4T1 growth observed with compound **4** alone was significantly abrogated (Figure 4). Avoiding

Figure 4. 4T1 cell proliferation in 3D culture. Growth inhibition occurs with treatment of 10 μM compound **4**. Addition of 3 μM of a specific c-Abl inhibitor (GNF-2) mitigates effects of compound **4**. * p-value < 0.005.

inhibition of off-target kinases that have anti-oncogenic activities is an important and frequently neglected advantage of selective inhibition. In addition to c-Abl, we anticipate there are other kinases inhibited by PP2 whose native activity is anti-oncogenic.

The results with 4T1 cellular inhibition highlight the potential utility of c-Src inhibitors in breast cancer therapy. Indeed, the identification of c-Src as a major resistance pathway to Herceptin therapy has reinvigorated the clinical exploration of c-Src inhibitors as a breast cancer therapy.[8] Unfortunately, there are no c-Src inhibitors in preclinical development or clinical use that are not also inhibitors of c-Abl.[4,9] Our data (and others[30,31]) indicate that inhibition of c-Abl should be avoided in breast cancer. Furthermore, development of highly selective c-Src inhibitors for therapeutic use should be pursued (rather than dual-Src/Abl and/or pan-kinase inhibitors).

Cancer cell profiling was performed by the National Cancer Institute (NCI 60 panel).[33] Consistent with published reports for inhibition of c-Src using genomic techniques,[29] compound **4** was cytostatic (and not cytotoxic). At a single concentration of 10 μM, mean growth across 57 cell lines tested was 71%. Seven cell lines showed <50% growth. There is good correlation between two of the cell lines, HT-29 and MCF7, where full dose–response curves were obtained in our laboratory (*vide supra*). For complete information on the NCI 60 panel with compound **4**, see the Supporting Information.

Along with dramatic increases in cellular efficacy against cancer cell lines, toxicity to a non-cancer cell line, NIH-3T3, was significantly reduced with selective c-Src inhibition (Table 2). With compound **4**, no growth inhibition up to 100 μM is observed, while PP2 has a GI_{50} of 17 μM for NIH-3T3 cells. PP2 is actually more effective at slowing the growth of non-cancerous NIH-3T3 cells than 3 of the 4 cancer cell lines examined. The reduced toxicity to healthy cells afforded by compound **4** is yet another advantage of selective inhibition.

Conclusion. Despite being the first oncogene discovered and playing a central role in many cancer signaling pathways, there have been no reports of a truly selective c-Src inhibitor that can be used in cellular studies. Using a novel approach of extending into the P-loop pocket of c-Src, we have developed a highly selective probe for c-Src activity. Moreover, we have shown distinct advantages to selective inhibition. Studies using compound **4** to improve our understanding of c-Src signaling in cancer are in progress.

METHODS

Synthesis of Compounds 1−6. Synthetic schemes, detailed procedures and characterization of compounds 1−6 can be found in the Supporting Information.

General Procedure for Biochemical Inhibition Assays. To determine inhibitor efficacy, we utilized a previously reported continuous, fluorimetric assay.[34] Briefly, phosphorylation of a self-reporting peptide substrate causes a fluorescence emission increase at 405 nm (ex. 340 nm). Final assay concentrations were as follows: ATP = 100 μM, substrate peptide = 45 μM. For more information and dose−response curves for each compound, see Supporting Information.

Production of c-Src-TM (Q275G, C277Q, F278Y). Chicken c-Src kinase domain in pET28a, modified with a TEV protease cleavable N-terminal 6x-His tag, was prepared as previously reported.[35] The desired mutations were added to this plasmid using iterative rounds of mutagenesis using the Agilent QuikChange II kit. The plasmid was transformed by electroporation into Bl21DE3 electrocompetent cells containing YopH in pCDFDuet-1. Cell growth, expression, and protein purification were performed using modified literature protocols for expression of wild-type c-Src kinase domain.[35]

Phospho-Erk Assay. SK-BR-3 cells (ATCC) were plated in 96-well plates at a density of $1.0−2.0 \times 10^4$ cells per well. The cells were grown to 80−90% confluency prior to overnight serum-starvation in DMEM, 0.1% BSA. The serum-free media was then removed and replaced with DMEM containing 100 μM compound 4 (or PP2) in 1% DMSO. The cells were incubated for 60 min prior to addition of EGF (Sigma Aldrich). After incubation, the media was removed, and 50 μL of AlphaScreen lysis buffer (PerkinElmer) was added to each well. The lysates were analyzed using the AlphaScreen SureFire Erk1/2 (p-Thr202/Tyr204) assay kit (PerkinElmer) according to the manufacturer's protocol.

Cell Growth Inhibition Assays. Cancer cell lines (obtained from ATCC) were dispersed from 70% to 80% confluent monolayer cultures using 0.25% trypsin-EDTA and plated in 96-well microtiter plates at $5.0−7.0 \times 10^3$ cells per well. The cells were allowed to attach for 24 h in medium (DMEM, 10% FBS, 1X pen/strep) at 37 °C in a humidified incubator with 5% CO_2. After 24 h, the growth medium was replaced with medium (DMEM, 10% FBS, no antibiotic) containing compound to be tested at 1% DMSO final concentration. After 72 h, WST-1 (Roche Applied Science) was added according to the manufacturer's procedure and incubated 60 min. After incubation, absorbance readings at 450 and 630 nm were taken. Data analysis and curve fitting was performed using Graphpad Prism software. See Supporting Information for dose−response curves.

4T1 Cell Growth in 3D Culture. 4T1 cells (ATCC) were cultured and assayed on Cultrex basement membrane extract (BME, Trevigen) cushions using the following procedure: 50 μL of BME was added to each well of a 96-well plate incubated on ice. The cushions were allowed to form at 37 °C for 30 min. 4T1 cells were grown to 70−80% confluency in growth media (DMEM, 10% FBS, no antibiotics) and dispersed using 0.25% trypsin-EDTA. The cells were plated on the cushions in growth media supplemented with 5% BME and allowed to grow for 72 h. Cell clusters were observed microscopically after 36 h. After 72 h, the media was removed and replaced with media containing compound in 1% DMSO. The cells were then cultured for an additional 72 h. After 72 h, WST-1 (Roche Applied Science) was added according to the manufacturer's procedure and incubated 60 min. After incubation, the medium was removed and added to fresh wells where the 450 and 630 nm absorbance was read.

ASSOCIATED CONTENT

Ⓢ Supporting Information

Supplementary figures, experimental methods, and characterization of compounds 1−6. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

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